

Seroprevalence of *Brucella abortus* in cattle at communal diptanks in the Mnisi area, Mpumalanga, South Africa

By

Nelson Matekwe

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Department of Veterinary Tropical Diseases

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Declaration

I, *Nelson Matekwe*, declare that the serum samples for this study were collected from cattle in the Mnisi district of Mpumalanga. The Rose Bengal tests, complement fixation tests and enzyme linked immunosorbent assays were done by the Hluvukani Animal Health Clinic, Ermelo Veterinary Laboratory and the Serology Section of the Department of Veterinary Tropical Diseases, respectively. Apart from the acknowledgements indicated and the advice and guidance by my supervisors, this dissertation is my own original work.

This full dissertation or any part of it has not been, is not being or will not be submitted for another degree at this or any other university.

This dissertation is presented in partial fulfilment for the requirements of a Master of Science Veterinary Tropical Diseases in the Department of Veterinary Tropical Diseases Faculty of Veterinary Science University of Pretoria.

Signed.....

Date.....

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Abbreviations

DAFF - Department of Agriculture Forestry and Fisheries

KNP – Kruger National Park

GLTCA - Great Limpopo Transfrontier Conservation Area

OIE – Office International des Epizooties (World Organisation for Animal Health)

ELISA – enzyme linked immunosorbent assay

iELISA – indirect enzyme linked immunosorbent assay

cELISA – competitive enzyme linked immunosorbent assay

RBT – Rose Bengal test

CFT – complement fixation test

FMD – foot and mouth disease

OPS – O-polysaccharide

sLPS – smooth-lipopolysaccharide

SAT - serum agglutination test

FPA – fluorescence polarisation assay

BBATs – buffered brucella antigen tests

BPAT - buffered plate agglutination test

MRT – milk ring test

S19 – strain 19

PCR - polymerase chain reaction

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By

Nelson Matekwe

Promoter: Dr JE Crafford

Department: Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria

Degree: Master of Science (Veterinary Tropical Diseases)

Abstract

This study determined the seroprevalence of *Brucella abortus* in cattle at communal diptanks in the Mnisi area of Mpumalanga, South Africa. A total of 1 482 serum samples was collected from cattle at 19 diptanks and tested for antibodies against *B. abortus* using the Rose Bengal test (RBT). A total of 21 cattle tested positive for *B. abortus* antibody on RBT. Thirteen of the 21 samples that tested positive for *B. abortus* on RBT also tested positive on complement fixation test (CFT) and 12 of these tested positive on indirect enzyme linked immunosorbent assay (iELISA). The overall CFT confirmed seroprevalence of brucellosis in Mnisi area was 0.88%. The seroprevalence varied from 0% to 3.49% between diptanks. Seven diptanks recorded 0% prevalence. Antibody to *B. abortus* was found to be most prevalent in cattle from diptanks near the central part of the Mnisi area.

Key words: Mnisi, cattle, *Brucella abortus*, seroprevalence, livestock-wildlife interface

Chapter 1

General introduction

Background

Brucellosis is a contagious, economically important disease of animals and man. Cattle brucellosis in particular is economically important due to: abortions in cows, infertility in cows and bulls, costs involved in control and eradication and the ban on international trade in cattle and dairy products. It is also of public health importance due to its ability to cause disease in humans.

The disease can readily be transmitted to humans often due to occupational exposure, essentially acquired through oral, respiratory or conjunctival routes. In endemic areas the consumption of dairy products constitutes the main risk to the general public. Brucellosis in humans is an acute febrile illness called undulant fever. The most common clinical features include fatigue, headache, sweating, joint pain, loss of appetite, muscular pain, lumber pain, weight loss, hepatomegally, splenomegally and arthritis. These multiple and non-specific features impede its diagnosis especially in areas where diseases with similar clinical features such as malaria, tuberculosis, typhoid and joint diseases co-exist ([Young 1995](#)). In rural communities such as Mnisi, the risk of cattle to human transmission is present due to the fact that most rural community dwellers do not pasteurize milk before consumption and animal handlers can handle aborted material. Studying the prevalence of brucellosis in Mnisi may therefore give an indirect estimate of the risk of possible exposure to humans.

The Mnisi area is a rural community with varying degrees of poverty, located on the central western border of the Kruger National Park (KNP). The cattle population in the area is approximately 17 000 and contributes through small scale subsistence farming to the livelihoods of the community. The reasons for keeping cattle are many, *i.e.* wealth accumulation (financial reserve/bank), draught power, manure, milk, ceremonial purposes, rituals and in African tradition for the payment of dowry in the form of lobola.

The fact that Mnisi shares its northern, eastern and southern borders with wildlife conservation areas poses a further risk for the transmission and maintenance of brucellosis between wildlife, livestock (in particular cattle) and humans, *i.e.* wildlife/livestock/human interface.

Cattle brucellosis is an OIE listed disease and a controlled disease in South Africa.

Apart from a study done in cattle in the rural communities of KwaZulu-Natal ([Hesterberg et al/ 2008](#)), the prevalence of bovine brucellosis in most rural communities of South Africa is unknown.

Studying the prevalence of brucellosis in Mnisi will provide important information regarding the current status of brucellosis in South Africa and the first step in determining the risks of possible exposure to humans and wildlife. Information from the study can be used to implement sustainable control measures to minimise animal to animal transmission as well as animal to human transmission of the disease.

Objectives of the study

To determine the seroprevalence of *Brucella abortus* in cattle in the Mnisi district of Mpumalanga using the Rose Bengal test, complement fixation test and indirect ELISA.

The results of this study will contribute important data with regard to the current status of brucellosis in South Africa. Knowledge of the apparent prevalence of brucellosis in cattle will also be invaluable for determining the risks of possible exposure of humans and wildlife. High apparent prevalence levels could alert the authorities to intensify control measures whereas negative herds will have an added value in terms of local trade in cattle and cattle products. This will encourage farmers to keep their herds free of brucellosis and therefore further reduce the prevalence of bovine brucellosis in the region.

Chapter 2

Literature Review

Introduction

It is suspected that bovine brucellosis was introduced into southern Africa by cattle that were imported from Europe ([Henning 1956](#)). The disease could also have been introduced by people and livestock migrating into southern Africa from other countries ([Madsen 1989](#)). In South Africa the first reliable record of the disease was by Gray in 1906 after an abortion storm in cattle near Johannesburg, though the occurrence of the disease was only confirmed by Hall following isolation of *B. abortus* from the stomach contents of an aborted bovine foetus in 1913 ([Henning 1956](#)). There is no reliable, accurate data on the actual prevalence of brucellosis in cattle in southern Africa due to the fact that reports are based on laboratory results which do not necessarily reflect the true picture on the ground ([Mcdermott & Arimi 2002](#)). This is because the available results are not from specific co-ordinated regional surveillances but from tests done for the purpose of trade, local surveillance or diagnosis. In South Africa, the prevalence of brucellosis in cattle has shown significant decline since 1968 when compulsory calf-hood vaccination was introduced. The national prevalence declined from 10.5% in 1976 to 6% in 1979 ([Bosman 1980](#)) and further declined to 1.95 in 1985 ([Faul & Bosman 1987](#)). There is no recent reliable data on the national prevalence of bovine brucellosis in South Africa.

Aetiology

Brucella abortus is the causative organism of contagious abortion in cattle. It is a small, gram positive, partially acidfast, non-sporulating, non-encapsulated coccus, coccobacillus or short rod of about 0.6-1.5 µm in length and 0.5-0.7 µm in width (Alton, Jones, Angus & Verger 1988). This species has smooth lipopolysaccharide (sLPS) on the cell surface which is considered a major virulence factor for disease (Porte, Naroen, Ouahrani-Bettache & Liautard 2003). *B. abortus* is a facultative intracellular bacterium (Nicoletti 1980). There are eight biovars, namely biovar 1, 2, 3, 4, 5, 6, 7 and 9 of which biovars 1 and 2 are the main ones encountered in South Africa. Biovar 1 is the most frequently isolated from cattle (Nicoletti 1980).

Epidemiology

Cattle are generally infected *per os* through ingestion of contaminated feed or water. Foetal membranes, uterine discharges and aborted fetuses are important sources of infection (Samartino & Enright 1993). Infection through the conjunctiva and inhalation of organisms have been documented. Calves can be infected vertically and through ingestion of infected milk (Nicoletti 1980). Some heifers born of seropositive dams may become latently infected and may remain serologically negative until the middle of their first gestation or later when they seroconvert for the first time (Catlin & Sheehan 1986). About 90% of infected cows may become chronically infected with the infection being confined to the udder and lymph nodes (Nicoletti 1980). Bulls that were infected *in utero* or *per os* during calf-hood may retain the infection until adult-hood (Rankin 1965). Infected bulls may shed *brucellae* in semen, seminal fluid and urine (Plant, Claxton, Pakovjevic & de Saram 1976).

The African buffalo (*Cyncerus caffer*), zebra (*Equus burchell*), eland (*Taurotragus oryx*), impala (*Aepyceros melampus*) and hippopotamus (*Hippopotamus amphibius*) have tested serologically positive for brucellosis. Serological surveys revealed up to 23% positive reactors in African buffalo from the Kruger National Park (KNP) of South Africa ([Herr & Marshall 1981](#)). The chances of wildlife and cattle mixing at the interface is minimal due to the well maintained fences and strict movement control measures to prevent the spread of foot and mouth disease (FMD) across the boundaries of the KNP as well as the adjoining private nature reserves ([Bengis, Kock & Fischer 2002](#)). However the risk of brucellosis being transmitted to cattle will increase when there is a break in the fence. In Zambia, a cross-sectional survey was carried out to investigate the risk factors associated with cattle herds reared in the livestock-wildlife interface areas of the Blue Lagoon and Lochinvar National Parks. The results of the study identified herd contact and sharing of pasture and water with wildlife as risks for cattle to seroconvert to *B. abortus* ([Muma et al 2007](#)).

Humans can become infected after ingestion of unpasteurised dairy products from infected cattle or after accidental self-injection with the *B. abortus* strain 19 (S19) vaccine. Bovine brucellosis is regarded as an occupational disease for veterinarians, laboratory personnel, stock inspectors and abattoir personnel ([Young 1995](#)).

Pathogenesis

The most common route of *B. abortus* infection in cattle is through the gastrointestinal tract. *B. abortus* invades the intestinal mucosa via M cells in the Payers patches and replicate intracellularly ([Gorvel & Moreno 2002](#)). Phagocytosed organisms are carried to regional lymph nodes where they replicate and cause lymphadenitis. Bacteraemia occurs which may persist for a few weeks. Apart from the lymph nodes, *B. abortus* also localise in

the spleen. In bulls it can localise in the accessory sex glands and the testicles and in cows in the udder and the pregnant uterus (Nicoletti 1980). The organism is also capable of invading trophoblastic cells (Carvalho-Neta *et al* 2010). Intracellular replication in the placental trophoblast is largely influenced by the stage of pregnancy and this usually occurs during the mid and late stage of pregnancy when the cells actively secrete steroid hormones (Samartino & Enright 1993). Localisation can also occur in the joints causing the typical hygromas seen in some infected cattle (Fensterbank 1978). *B. abortus* causes hormonal changes in the infected placenta by reducing the levels of progesterone and increasing the levels of cortisol, oestrogen and prostaglandin $F_{2\alpha}$ (Gorvel & Moreno 2002). These hormonal changes are likely to be the major contributing factor to abortion which occurs as a result of *B. abortus* infection (Carvalho-Neta *et al* 2010).

Following exposure, innate immunity plays a pivotal role in decreasing the infective dose at the site of entry as well as inhibiting the intracellular growth of *B. abortus*. Dendritic cell activation during infection with *B. abortus* induces T-cells to produce interferon gamma (Copin *et al* 2007).

Clinical signs

Abortion is the main clinical sign in a herd infected with bovine brucellosis. Abortions may be accompanied by a significant reduction in milk production (Crawford, Huber & Adams 1990). Chronically infected animals may develop hygromas (regarded as highly indicative of infection) which may be unilateral or bilateral and infected bulls may develop unilateral or bilateral orchitis, epididymitis and seminal vasculitis which may be acute or chronic (Bishop, Bosman & Herr 1994). Infected bulls can develop permanent sterility due to

chronic orchitis and fibrosis of the testicular parenchyma ([Campero et al 1990](#)). Clinical signs are not always apparent and bulls may not show any clinical signs at all.

Bulls vaccinated with the S19 vaccine develop orchitis ([Bishop et al 1994](#)). A progressive, erosive, non-suppurative arthritis of the stifle joints was reported in brucellosis-free cattle that were vaccinated with the S19 vaccine ([Wynn-Jones, Baker & Johnson 1980](#)).

The incubation period of bovine brucellosis is variable with an average of 225 days for cows infected at service. Cows infected at seven months of gestation usually abort 50 days later ([Thomson 1950](#)). After the first abortion due to brucellosis cows may have normal pregnancies although subsequent abortions may occur. Vertically infected calves may remain seronegative without showing any signs of the disease ([Nicoletti 1980](#)). The longest recorded incubation period in a cow is nine years ([La Praik et al 1975](#)).

Diagnosis

Diagnosis of cattle brucellosis is based on three groups of tests, i.e. tests that demonstrate the organism, tests that demonstrate allergic reactions to *B. abortus* and tests that detect antibodies.

Tests that demonstrate *B. abortus* organisms

Microscopic examination

On tissue section and smears *B. abortus* stains red against a blue background using the Stamp's modification of the Ziehl-Neelsen stain ([Stamp et al 1950](#)).

Polymerase chain reaction (PCR)

This is a technique used to detect the nucleic acid of the *B. abortus* organism. This method of diagnosis can differentiate between some of the biovars by using PCR restriction fragment length polymorphism and southern blot. PCR is also used in biotyping and distinguishing vaccine stains ([OIE Terrestrial Manual 2009](#)).

Culture and typing

B. abortus can be cultured and typed from specimens of the lung, placenta, liver, spleen and stomach contents of an aborted foetus. In the live cow it can be cultured from uterine discharges, milk and colostrum. At slaughter, the supramammary lymph node is the most suitable although other lymph nodes such as the retropharyngeal, parotid, mandibular, prescapular and iliac can also be used for culture ([Alton et al 1988](#)).

Tests to detect an allergic reaction to *B. abortus*.

Brucellin skin test

This test is an alternative immunological test for screening unvaccinated herds ([OIE Terrestrial Manual 2009](#)). It is a very specific test. The test can detect infection in sero-negative cattle ([Bercovich et al 1992](#)) and can also detect latent *B. abortus* infections ([Muskens, Bercovich & Damen 1995](#)). The main advantage of the test is that it can be used to rule out false positive serological reaction due to infection with *Yersinia enterocolitica* O9 ([Bercovich et al 1992](#)). Other advantages are that there is no need for laboratory facilities to carry out the test and that it is an ideal test method to use in circumstances where animal identification is difficult, e.g. cattle raised under pastoral conditions. The main disadvantage is that it is more laborious compared to the serological

tests as the animals have to be handled twice (measuring skin thickness pre and post injection of Brucellin).

Tests for the detection of specific *B. abortus* antibody

There are a variety of serological methods described for the diagnosis of *B. abortus* in cattle. Most of them are based on detecting specific antibody directed against the O-polysaccharide (OPS) side chains of the smooth lipopolysaccharide (sLPS) component in the outer membrane of the organism ([Nielsen & Ewalt 2004](#)).

There are however some limitations in these assays, one being that cattle vaccinated with attenuated *B. abortus* strain 19 have residual antibody specific for sLPS which cannot be distinguished from that resulting from field strain infection ([World Health Organisation 1971](#)) and the other being that the sLPS of bacteria such as *Yersinia enterocolitica* 0:9 ([Lindberg et al 1982](#)) *Vibrio cholerae* ([Sandulache & Marx 1978](#)) *Escherichia coli* ([Nielsen, Samagh & Stemshorn 1980](#)) and *Salmonella* Kauffmann-White Group N ([Corbel 1975](#)) cross react with *B. abortus* leading to false positive reactions.

Following infection with *B. abortus* in cattle, the early antibody response consists predominantly of an IgM response. The duration of the response is based on factors such as the dose of bacteria, route of infection as well as the health status of the animal. Production of IgG follows the IgM response and later small quantities of IgG₂ and IgA appear. Assays that predominantly measure IgG₁ are the most useful in the diagnosis of *B. abortus*. This is because most cross reacting antibodies generated from exposure to other organisms and environmental antigens are IgM ([Allan, Chappel, Williamson & McNaught 1976](#)).

Classical serological tests currently employed by the Department of Agriculture Forestry and Fisheries (DAFF) of South Africa are the Rose Bengal test (RBT), the complement fixation test (CFT) and the serum agglutination test (SAT). The RBT is used for screening purposes while the CFT is used as a confirmatory test. The SAT is only occasionally used as it has some value to detect IgM which is the predominant immunoglobulin after S19 vaccination. No single test is able to detect all the various antibody isotypes ([Nielsen, Wright, Kelly & Cherwonogrodzky 1988](#)).

The OIE recommends the buffered *Brucella* antigen tests (BBATs) as well as the ELISA and the fluorescence polarisation assay (FPA) as suitable screening tests for the control of brucellosis at national or local level. The BBATs include the RBT and the buffered plate agglutination test (BPAT). Positive reactions should be retested with a suitable confirmatory test.

1 Buffered *Brucella* antigen tests

1.1 Rose Bengal test (RBT) (acidified antigen)

RBT uses *B. abortus* s99 or s1119.3 whole cells, stained with Rose-Bengal. RBT detects three of the four antibody isotypes, *i.e.* IgM, IgG₁ and IgG₂. It is therefore commonly used as a screening test due to its high sensitivity, followed by the CFT (detects IgG₁) which has a high specificity as a confirmatory test. This test is quite sensitive for the detection of vaccine induced antibodies, though false negative results may occur due to prozoning which may be overcome by diluting the serum or retesting after a certain period of time ([OIE manual 2009](#), [Nielsen *et al* 1996](#)). The test is standardized and is quite simple to perform and is relatively cheap. Antibodies resulting from *B. abortus* S19 vaccination and

some cross reacting antibodies are detected by this test therefore it is necessary to use other tests to confirm reactor animals as infected ([OIE manual 2009](#)).

1.2 Buffered plate agglutination test

This test is quite similar to the RBT in terms of sensitivity as well as the detection of vaccine induced antibody. Like the RBT, positive samples on BPAT must be retested using a confirmatory test. False negative results may occur due to prozoning as with RBT ([OIE manual 2009](#)).

2 Complement fixation test (CFT)

Dilutions of serum from cattle and pre-titrated amounts of complement and antigen are added together. Guinea pig serum is used as a source of complement. Indigenous complement in the test serum is inactivated first by heating for 30-60 min at 58 ± 2 °C. If antibody is present in the test serum, it will bind to the antigen. If the antibody is of the IgG₁ isotype, the complement will be bound and fixed by the antigen-antibody complex.. An indicator system that consists of sheep erythrocytes sensitized with rabbit antibody is then added. In the event of a *Brucella* positive test serum there would be no available complement to cause lysis to the erythrocytes. Alternatively, with a *Brucella* negative test serum, the available complement will be activated by interaction with receptors on the Fc portion of the rabbit antibody and will cause lysis of the red blood cells. This will release haemoglobin which can be assessed visually or by a spectrophotometer.

There are two variations of the CFT standardized by Hill ([1963](#)) and MacKinnon ([1963](#)). The procedures are basically the same varying only on the incubation temperatures and time, either 37 °C for 1 h or 4 °C for 18h. The main shortfall of the CFT is its inability to distinguish between vaccine reactions and field infection. There is also the occasional

occurrence of serum samples that activate complement in the absence of antigen. CFT is recommended by the OIE as a prescribed test for international trade ([Nielsen 2002](#)). CFT does not detect IgG₂.

3 Enzyme linked immunosorbent assay (ELISA)

Several ELISAs have been developed over the years for the detection of both antigen and antibody in milk and serum. The aim was to develop assays that could overcome the limitations of the conventional tests not detecting one or more of the relevant antibody isotypes. Some of these assays were reviewed by Nielsen ([1988](#)). A variety of antigen preparations ranging from whole cells to purified components were used. Most of the assays used soluble antigen mixtures of which sLPS was probably the most important antigenic determinant ([Nielsen et al 1988](#)).

3.1 Indirect enzyme immunoassay (iELISA)

The first iELISA was developed for the diagnosis of human brucellosis ([Carlsson et al 1976](#)). Since then several variations using different antigens, antiglobulin-enzyme conjugates and substrate chromogens were developed. The problem with false positive reactions may be partly overcome by using rough LPS or cytosol antigens. Monoclonal antiglobulin-, polyclonal antiglobulin- or protein G- or protein A enzyme conjugates have been used. The specificity of the assay can be improved by the use of a monoclonal antibody specific for the heavy chain of bovine IgG₁, however this may lead to possible loss of sensitivity. The OIE currently recommends purified sLPS as the antigen, serum diluted 1:50 and mouse monoclonal antibody specific for bovine IgG₁ conjugated with horseradish peroxidase ([OIE Manual 2009](#)). The disadvantage of the iELISA is its inability

to distinguish antibody resulting from vaccination with *B. abortus* S19 from that resulting from field strain infection ([Nielsen & Gall 1994](#)).

3.2 Competitive enzyme immunoassays (cELISA)

This test was developed to overcome the failure by the other serological tests to distinguish vaccinal antibody. A competing antibody can be selected to inhibit the binding of vaccinal but not the field strain antibody ([Nielsen 2002](#)). The most commonly used format of the cELISA utilizes sLPS from *B. abortus*, passively adsorbed to polystyrene matrix followed by incubation with competing antibody and appropriately diluted serum. After mixing and incubation, a reagent for detecting bound monoclonal antibody, labelled with an enzyme, usually horseradish peroxidase or alkaline phosphatase, is added followed by substrate/chromogen after a suitable incubation period. A wash procedure is performed between each step. A series of controls, including a strong positive, a weak positive, a negative serum as well as buffer (no serum) must be included. Results are calculated as percentage inhibition against the buffer control (0% inhibition). The cELISA is a prescribed test by the OIE for international trade of cattle ([OIE Manual 2009](#)).

4 Fluorescence polarisation assay (FPA)

FPA is a rapid and simple test that can be performed both in the field (using a portable analyser which receives power from a laptop computer) and in the laboratory. The test measures the interaction between the antigen and the antibody ([Nielsen & Gall 2001](#)). It is an alternative OIE test for bovine brucellosis ([OIE Manual 2009](#)). The principle of the test is that a molecule (labelled with a fluorescence maker) in solution randomly rotates at a rate inversely proportional to its size and is examined by plane polarised light. The binding of the antibody to the fluorescing antigen influences the size of the molecule and therefore

the polarisation of the light *i.e.* a small molecule will rotate faster than a large molecule. A fluorescence polarisation analyser is used to acquire a measurement of fluorescence of the diluted serum sample. The results are presented in millipolarisation units ([Nielsen 2002](#)).

5 Other tests

5.1 Serum agglutination test (SAT)

The SAT is not a very specific test though its specificity may be improved by the addition of EDTA (5mM) to the antigen suspension. The test is particularly used to detect new infections as early as two weeks post infection. However its use in chronically infected animals is quite limited because of false negative results ([Godfroid & Kasbohrer 2002](#)). SAT is usually used as a supplementary test for determining the level of IgM, which is the predominant immunoglobulin produced after vaccination with the S19 vaccine ([Herr & Brugge 1985](#)).

5.2 Milk ring test (MRT)

This is an agglutination test that uses hematoxylin stained whole cell antigen added to milk ([Huber & Nicoletti 1986](#)). If there are antibodies in the milk the Fc portion of the antibodies will be attached to the milk fat globules. The antibodies agglutinate with the antigen and as the fat globules rise a purple band will appear at the top of the milk. In the absence of antibodies the fat band will remain uncoloured. This test can be used as a screening test of bulk milk samples of lactating cows. In herds with more than one hundred cattle, the sensitivity of the MRT becomes less reliable and the volume of the test sample should be adjusted according to the following formula: herd size < 150 animals

use 1 ml bulk milk, 150-450 use 2 ml milk sample, 451-700 use 3 ml milk sample ([OIE manual 2009](#)).

5.3 Interferon gamma test

The test involves stimulation of lymphocytes in whole blood with brucellin and then measuring the resulting gamma interferon produced by a capture ELISA. This test is used when the prevalence of brucellosis is low as it is useful in discriminating against false positive serological reactors ([OIE Terrestrial manual 2009](#)).

5.4 Native hapten and cytosol protein based tests

These tests have been used in combination with RBT as screening tests in brucellosis eradication programmes. The tests are highly specific in S19 vaccinated cattle ([OIE Terrestrial manual 2009](#)).

Chapter 3

Materials and methods

Study area

This study was carried out in the Mnisi communal area of Mpumalanga Province of South Africa. The area shares borders with nature reserves on three sides; Timbavati to the north, Manyeleti to the east and Sabi Sand to the south (see Figure 1 below). There is communal land to the west of the study area which is used for subsistence farming. The nature reserves are mainly for tourism.

Well maintained fences separate the nature reserves and Mnisi communal area. However the possibility of direct interaction between wildlife and livestock populations at the interface cannot be ruled out.

The farmers make use of communal grazing where animals are released during the day to fend for grazing and water and then rounded up and kraaled during the night for security purposes as well as production of manure. Each farmer kraals his herd of cattle near his homestead. Cattle from 19 diptanks (see Table 1 for names and GPS co-ordinates) participated in the survey. The diptanks are clearly indicated on the map of the study area below (Figure 1). According to the current cattle census records from the Department of Veterinary Services in Mnisi, the cattle population in the area is 16 418 and the total number of cattle owners is 1 497. This gives an average number of 11 head of cattle per farmer/herd. The human population in the area is about 40 000. The proportion of human to cattle population is approximately 2 people per cow.

Table 1 Tabulated GPS coordinates of the diptanks in Mnisi.

Dip tank	Latitude	Longitude
Athol	-24.713611	31.352222
Burlington	-24.644167	31.213889
Clare A	-24.628889	31.305556
Clare B	-24.647222	31.331111
Eglington	-24.653611	31.342222
Gottenburg	-24.638333	31.415833
Hlalakahle	-24.617222	31.433889
Islington	-24.622778	31.259167
Ludlow A	-24.653611	31.281111
Ludlow B	-24.655000	31.282500
Seville A	-24.679444	31.404444
Seville B	-24.674167	31.441389
Share	-24.681667	31.315833
Shorty	-24.675556	31.374722
Tlhavekisa	-24.627500	31.380278
Utha A	-24.699722	31.445556
Utha Scheme	-24.710833	31.401667
Welverdiend A	-24.583333	31.325556
Welverdiend B	-24.578333	31.359444

About 71% of farmers do not use a specific bull for breeding with their cows and about 61% indicated that they are satisfied with the quality of bulls that they have ([Van Rooyen et al 2009](#)). Since animals make use of communal grazing, this means that individual herds belonging to the same diptank may not necessarily be considered as closed herds and therefore bulls may be shared between herds.

Farmers take their animals weekly to the diptanks for compulsory plunge dipping against ectoparasites. Each dipping session is supervised by an animal health technician. This offers a good opportunity for the animal health technician to inspect the cattle for foot and mouth disease (FMD). During each dipping session cattle registers are updated (new born

calves and newly introduced cattle are added and those sold, slaughtered or dead are removed from the register).

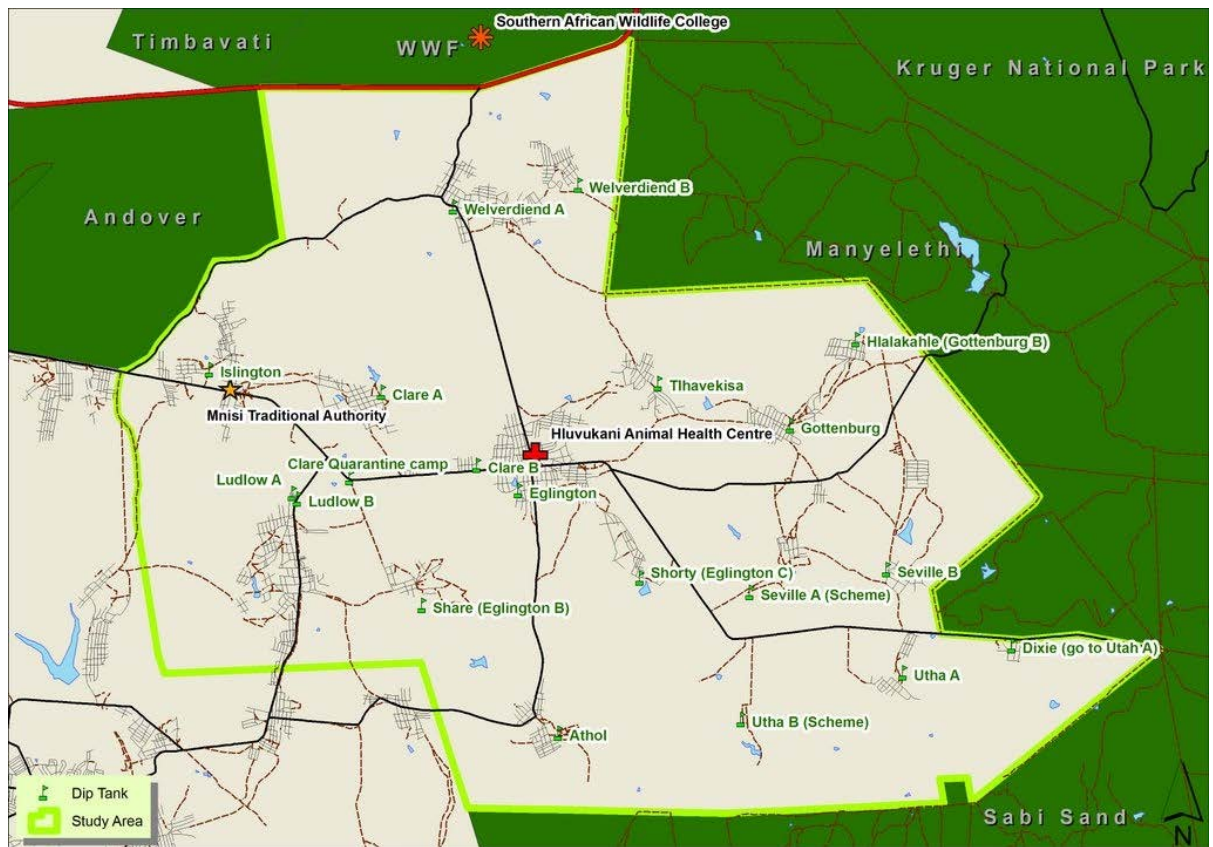


Figure 1 Map of Mnisi study area showing the location of the diptanks and surrounding nature reserves.

Study population

Cattle in the study population represented a mixture of both *Bos indicus* and *Bos taurus* breeds. At the time of the study the general body condition of cattle was good.

Animals were individually identified according to the stock number, name of owner, colour, sex and age as well as the name of the dip tank to which the animal belonged.

In a survey done in the year 2009 on 10 of the 19 dip tanks, the proportion of cattle per herd was 13, i.e. 2 bulls, 6 cows, 3 heifers, 2 calves, 1 ox ([Van Rooyen et al 2009](#)).

Sampling methods

The prevalence of cattle brucellosis in Africa varies from 1.45% to 25% ([Muma et al 2007](#), [Hersterberg et al 2008](#)). Based on this information, an assumed prevalence of 1.5% was considered for this study. Using the formula by Daniel ([1999](#)) to calculate sample size for prevalence studies, with a precision of ± 7.5 and a 95% confidence interval, 10% of the Mnisi cattle had to be sampled. This was done by collecting blood samples from every 10th animal (systematic random sampling) coming through the crush during dipping time, regardless of age and sex.

However, it was difficult to attain 100% cattle attendance at the diptanks due to reasons beyond our control (farmer not available to bring the cattle for dipping, cattle not fetched or found from pastures and therefore misses the dipping session, etc.). This resulted in under sampling, i.e. 1 482 (9%) cattle were sampled instead of 1 642 (10%).

The cattle were bled from the tail vein using 20-gauge needles and sterile evacuated SST tubes (Greiner Bio-One GmbH). During field collection the blood tubes were clearly marked indicating the dip tank and sample number. Blood was allowed to clot for 3 hours at room temperature after which the blood tubes were centrifuged at 4000 g for 15 minutes. The sera were aliquoted into 1.2 ml cryotubes (Whitehead Scientific) and stored at -20 °C until tested. The cryotubes were clearly marked using a label that indicated the date of collection, name of diptank and sample number.

Table 2 A tabulated summary of the number of animals sampled for cattle brucellosis in Mnisi area. The table shows the population of cattle, number of cattle owners and the number of cattle sampled per dip tank.

Name of diptank	Code of diptank	No. of cattle owners	No. of cattle	No. of cattle sampled
Clare B	A	84	943	86
Welverdiend A	B	135	1648	152
Serville A	C	50	755	81
Thlavekisa	D	58	556	54
Gotternburg	E	99	963	103
Clare A	F	57	706	58
Utha Scheme	G	11	585	59
Welverdiend B	H	71	786	71
Athol	I	100	990	99
Eglington	J	123	1092	103
Share	K	56	709	62
Serville B	L	52	850	64
Shorty	M	46	545	55
Hlalakahle	N	47	436	45
Utha	O	81	812	76
Ludlow A	P	100	1069	90
Islington	Q	105	914	61
Burlington	R	118	1112	83
Ludlow B	S	104	947	80
Totals	19 dip tanks	1 497	16 418	1 482

Laboratory testing

Rose Bengal Test

Tests were done by a registered veterinarian at Hluvukani Animal Health Clinic located in the Mnisi area of Mpumalanga province. Onderstepoort biological products (OBP) *B. abortus* RBT antigen and OBP *Brucella* positive serum was used. The test was performed

in plates where equal volumes (25 micro litres) of serum and antigen were mixed and the tray rotated for 4 minutes at room temperature before reading the results.

Complement Fixation Test

Tests were performed by a registered veterinary technologist at the Mpumalanga Veterinary Laboratory (MPVL) in Emmerlo. This is a Department of Agriculture, Forestry and fisheries (DAFF) approved laboratory. Their approval number is DAFF-28 and the protocol name and number for their CFT is “*Brucella abortus* complement fixation test (MPVL STM SERO 002)”.

In brief the CFT procedure was as follows; the test sera were inactivated at 58 °C for 60 min in a water bath. Twenty-five micro litres Veronal Buffer (in house prepared) was dispensed into wells of columns 1 to 8 and row A to H in U-bottom microtitre plates. Fifty micro litres of inactivated undiluted samples were transferred into rows A1 to A8 (the two wells 7 and 8 were used for negative and positive controls). 25 µl of serum was transferred from row A and serial doubling dilutions were done to row H and the remaining 25 µl was discarded. 50 µl of complement (Guinea pig complement, Siemens) was dispensed to row B, wells B1-B8. 50 µl of *B abortus* antigen (equal volumes of antigen and complement) was dispensed to row C-H. The plates were incubated at 37 °C for 30minutes. After incubation 50 µl of the haemolytic system was dispensed to row A-H. The plate was placed on a shaker and incubated for a further 30minutes at 37 °C. After incubation the plates were removed from the incubator and centrifuge at 1000g for 1 minute. The plates were read over a magnifying glass as 0-4(0=100% haemolysis and 4=0% haemolysis). A negative result is where no sedimentation of red blood cells is

present *i.e.* complete haemolysis. A positive result is where sedimentation of red blood cells is present as a distinct button at the bottom of the well.

	1	2	3	4	5	6	7	8
A							N	P
B								
C								
D								
E								
F								
G								
H								

N= Negative control, P= Positive control

Figure 2 Plate layout for the CFT - indicating the positive, negative and sample positions.

ELISA

The commercially available *Brucella* kit “Pourquier ELISA Brucellosis Individual and Pool Serum Screening – Version Po4130/10” (Pourquier Institute, France) was used to test the cattle serum samples for *B. abortus* antibodies.

The ELISA test was performed according to the manufacturer’s instructions. Briefly, the kit includes microtitre plates pre-coated with *B. abortus* lipopolysaccharide antigen.

Serum samples to be tested (controls and samples) were diluted to 1/20 by dispensing 190 µl of dilution buffer to each well, 10 µl of negative control in well A1, 10 µl of positive control in well B1 and well C1, 10 µl of each serum sample to be tested per well as

indicated below (figure 3). The plates were covered with a lid and incubated on an orbital shaker for one hour on the bench to allow for the binding of the *B. abortus* reactive antibody in the test sample to the antigen in the wells. After incubation the contents of the plate were emptied and each microtitre plate was washed three times using the wash solution. 100 µl of diluted (1:100) conjugate (monoclonal anti-ruminant IgG-peroxidase conjugate) was added to each well and the plate was covered with a lid and incubated for 30 minutes on the bench. After incubation the plate was again emptied and washed as before. After washing 100 µl of the substrate was added to each well and the plate was incubated for 20 minutes in the dark after which 100 µl of the stop solution was dispensed into all the wells.

The degree of colour change that develops is directly proportional to the amount of antibody specific for *B. abortus* in the sample. The optical densities (OD) were read at 450 nm using an Biotech EL 808 ELISA reader.

The strength of each serum sample was expressed as a percentage positivity (PP) in relation to the positive control using the following calculation:

$$PP = (OD_{450_{\text{sample}}} - OD_{450_{\text{negative control}}}) / (OD_{450_{\text{positive control}}} - OD_{450_{\text{negative control}}}) \times 100$$

Sera with a PP value equal to or lower than 110% were considered to be negative. Sera with values between 110% and 120% were considered to be doubtful. Serum samples with values equal to or higher than 120% were considered positive for antibodies to *B. abortus*.

	1	2	3	4	5	6	7	8	9	10	11	12
A	N											
B	P											
C	P											
D	1											
E	2											
F	3											
G	4											
H	5											

N= Negative control, P= Positive control, 1 = Sample number 1

Figure 3 Plate layout for the ELISA - indicating the positive, negative and sample positions

Statistical analysis

All the serological data was captured on Microsoft excel spreadsheets. Apparent prevalence rates were calculated per diptank for each test.

Chapter 4

Results

In this study a total of 1 482 serum samples were collected from 19 diptanks with a total population of 16 418 cattle. The results indicated that the average apparent prevalence of *B. abortus* in Mnisi cattle was 0.88%. The apparent prevalence between the dip tanks ranged from 0% to 3.49%, with seven dip tanks showing no positive animals.

A total of 21 cattle tested positive for *B. abortus* antibody on RBT. These samples were subjected to CFT and ELISA tests. Thirteen of the 21 samples (62%) that tested positive for *B. abortus* on RBT also tested positive on CFT and 12 (57%) tested positive on ELISA. Ninety two percent (12/13) of the CFT positives were also positive on ELISA. Only one sample from Share diptank tested positive on CFT but negative on ELISA (Table 3), which could have been a false negative or the CFT could have been a false positive.

Because the CFT and ELISA results were in agreement the ELISA results were used on spatial distribution and mapping individual diptank prevalence values using ArcMap version 9.3 ArcGIS 9®software (figure 4 and 5).

The RBT apparent prevalence distribution among the cattle population was three heifers, three bulls and 15 cows of which two heifers, two bulls and nine cows tested positive by CFT and two heifers, two bulls and eight cows tested positive by ELISA.

Table 3 This table shows the RBT prevalence of cattle brucellosis per dip tank. Also indicated is the sample identification number and sex distribution of animals positive per test.

Name of dip tank	RBT prevalence	RBT positives (sample ID nr) and sex distribution of positives	RBT positives also positive on CFT (sample ID nr) and sex distribution of positives	RBT positives also positive on ELISA (sample ID nr) and sex distribution of positives
Clare B	3.49	3 (43,46,58) 1M, 2F	3(43, 46,58) 1M, 2F	3(43,46, 58) 1M, 2F
Welverdiend A	0	0	0	0
Servile A	2.47	2 (14,47) 1M, 1F	2 (14,47) 1M, 1F	2(14,47) 1M, 1F
Thlavekisa	1.85	1(42) F	1(42) 1F	1(42) 2F
Gotternburg	1.94	2(37,52) 2F	2(37,52) 2F	2(37,52) 2F
Clare A	0	0	0	0
Utha Scheme	1.69	1(42) F	0	0
Welverdiend B	1.41	1(45) F	1 (45) F	1(45) 1F
Athol	0	0	0	0
Eglington	1.94	2 (80,102)1M,1F	1 (102) F	1(102) F
Share	4.84	3(26,36,45) 3F	1 (26) F	0
Serville B	1.56	1(41) F	1 (41) F	1(41) F
Shorty	1.82	1(22) F	0	0
Hlalakahle	2.22	1(13) F	0	0
Utha	3.95	3 (21,11,7) 3F	1(11) F	1(11)
Ludlow A	0	0	0	0
Islington	0	0	0	0
Burlington	0	0	0	0
Ludlow B	0	0	0	0
Totals		21	13	12

Key; M= Male, F= Female

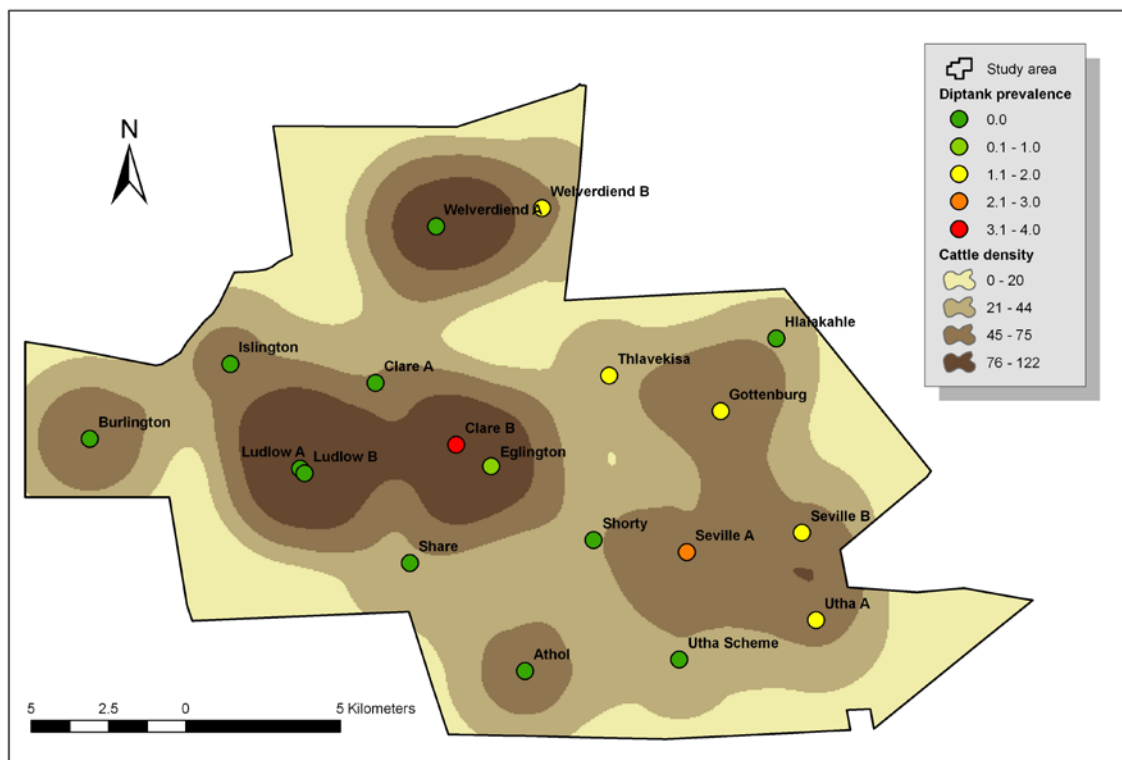


Figure 4 Map indicating the seroprevalence of *B. abortus* per diptank together with cattle density. The cattle density was plotted using the Kernal density method with a search radius of 4 km and represents number of cattle per km². The map was generated using ArcMap version 9.3 ArcGIS 9®software

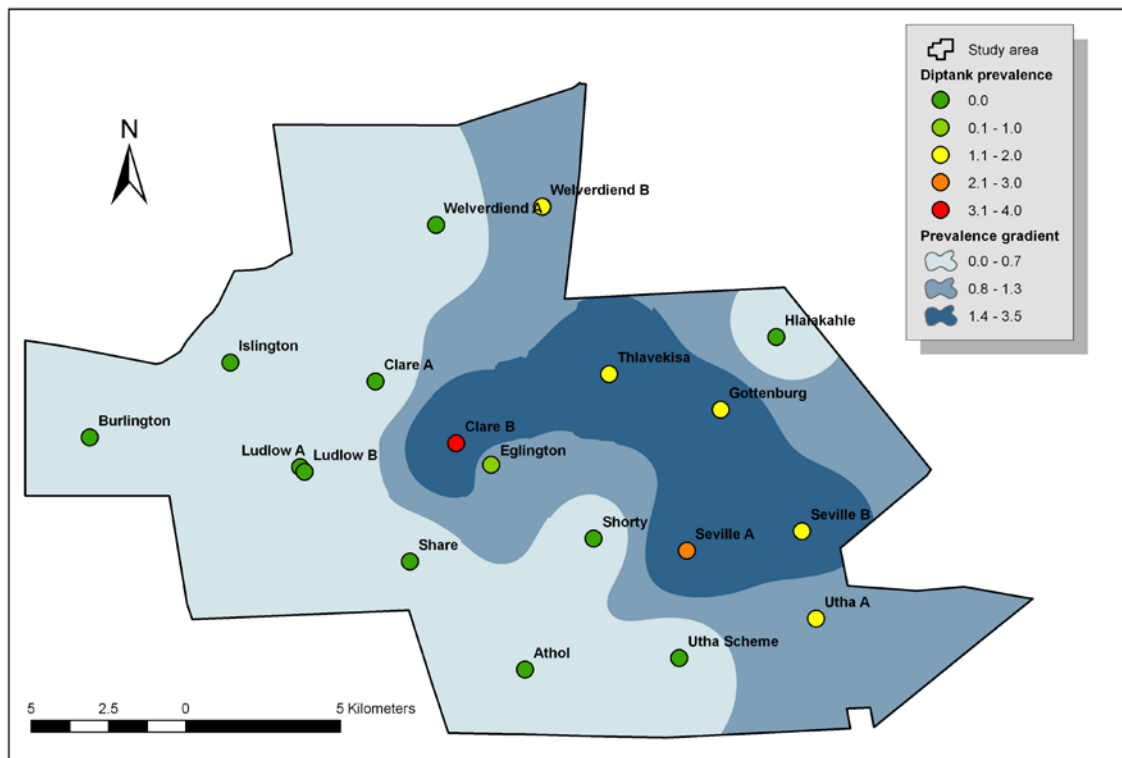


Figure 5 Map indicating areas of similar prevalence calculated through interpolation of the individual diptank prevalence values. The map was generated using the inverse distance weighed method in ArcMap version 9.3 ArcGIS 9[®] software.

Chapter 5

Discussion

This study was done to determine the sero-prevalence of cattle brucellosis in Mnisi. The results indicated that the average apparent prevalence of *B. abortus* in Mnisi cattle was 0.88%. The apparent prevalence between the dip tanks ranged from 0% to 3.49%, with seven dip tanks showing no positive animals. As mentioned under results (chapter 4), 9% of the cattle were sampled instead of 10% due to circumstances beyond our control. This may therefore mean that the determined apparent prevalence may not be absolutely correct.

As part of the DAFF recommendations for the control of cattle brucellosis, all heifers should be vaccinated once off at the age of 4 to 8 months with the S19 vaccine strain. All the cattle that tested positive for *B. abortus* antibodies were 2 years or older, except 3 which were all aged 1 year. The possibility of vaccine generated antibody being detected by the serological tests used in this study is therefore unlikely. However, the possibility that some heifers could have been older than 8 months at the time of vaccination cannot be totally excluded.

In most cases, interpreting serological surveys of diseases is difficult because most diagnostic or screening tests have imperfect sensitivity and specificity. Thus, there is a distinction between true prevalence (the proportion of the population that is actually infected) and the apparent prevalence (the proportion of the population that tests positive for the disease). Given point estimates for sensitivity (se) and specificity (sp), and

apparent prevalence (AP), one may calculate the true prevalence of the disease using the expression: $\text{True prevalence} = (\text{AP} + \text{sp} - 1) / (\text{se} + \text{sp} - 1)$

Studies by McDermot and Arimi (2002) found that cattle brucellosis is present in all main livestock production systems in sub-Saharan Africa. Serological prevalence was found to range from 4.8% - 41% in pastoral and livestock-subsistence crop farming systems in the semi arid areas (McDermott & Arimi 2002). As indicated above the mean apparent prevalence of brucellosis in Mnisi cattle is 0.88%. This is significantly low compared to the findings by McDermot and Arimi (2002).

Since the Mnisi communal area share borders with nature reserves, the population of wildlife in the nature reserves play an integral role in the health status of livestock at the wildlife interface. Wildlife populations may serve as reservoirs for infectious diseases and may have a negative impact on the well being and health status of livestock and humans at the interface.

A recent study carried out at the wildlife-livestock interface in the southeast lowveld of Zimbabwe, part of the Great Limpopo Transfrontier Conservation Area (GLTCA) where a similar cattle rearing system to Mnisi area is practiced, revealed cattle brucellosis prevalence to be 9.9% in a total of 1 158 cattle that were tested using RBT and cELISA. Their findings also revealed that seropositivity of cows was significantly associated with a history of grazing in the GLTCA (Gomo *et al* 2011). Their findings indicated a significant higher prevalence (9.9%) than this study (0.88%).

According to the OIE, bovine brucellosis status of South Africa for the period 1996 to 2004, a total of 3 154 outbreaks involving 51 279 cases were reported (OIE [handistatus 2](http://web.oie.int/hs2/) <http://web.oie.int/hs2/>). This gives an average of 350 outbreaks and 5 698 cases for South

Africa per year for that period of time. For the same period of time (1996-2004), the average cattle population was 13.5 million (<http://www.fao.org/ag/AGP/AGPC/doc/Counprof/southafrica/SouthAfrica.htm>). Based on the cases of bovine brucellosis reported to the OIE over this nine year period, the average national prevalence of the disease was 0.042%. This is probably an under estimation since not all cases are reported to the authorities. There is no reliable data on the cases of bovine brucellosis post 2004. An important factor to consider here is that the information is not derived from a co-ordinated national surveillance or prevalence study of the disease, but rather from tests done on cattle for the purposes of diagnosis, fertility tests, local and international movement of live cattle, as well as export of embryos and semen. However if information about the disease status in South Africa during the period 2005 to 2011 becomes available, it should be compared to the findings of this study.

The results of this study is in agreement with the results of a similar study carried out in communal grazing areas of Kwazulu-Natal province of South Africa between March 2001 and December 2003, where a mean prevalence of 1.45% was found after a total number of 46 025 cattle from 446 diptanks were tested using the RBT and CFT ([Hesterberg et al 2008](#)). Also worth noting is a brucellosis prevalence study carried out on 5 000 adult beef cattle that were slaughtered at an abattoir in Kwazulu-Natal where the prevalence was found to be less than 1.5% ([Bishop 1984](#)). The low prevalence or the absence of brucellosis at some dip tanks in Mnisi may be due to the fact that the prevalence of the disease is very low in the area. Though the contact of herds from different diptanks is regarded as minimal, the shortage of grazing, as is the case with Ludlow for example, cattle may encroach into neighbouring diptank grazing areas.

The relatively high prevalence found at the dip tanks Hlalakahle, Gotternberg and Utah A, which are located close to the Manyeleti nature reserves could not be explained. A study carried out in Zambia to assess the risk factors for brucellosis in cattle reared at the livestock/wildlife interface revealed that most of the sero-positive animals were from herds close to the national parks, where herds were likely to co-graze with wildlife ([Muma *et al.* 2007](#)). However no recent breakdown on the fence separating these dip tank areas from Manyeleti nature reserve has been recorded prior to this study. The bovine brucellosis status of wildlife in the Manyeleti nature reserve is unknown and therefore the possibility of wildlife acting as reservoirs for the disease could not be justified. However, it is recommended that a study be carried out to determine the *B. abortus* status of wildlife in the nature reserve. The study will provide information about whether cattle are at risk of being infected by brucellosis from wildlife or vice-versa. Another study carried out in Zambia recently revealed that the Kafue lechwe (*Kobus leche kafuensis*) antelopes which grazed in the same area with cattle were sero-positive (42.9%) to *Brucella* while the other antelopes, Black lechwe (*Kobus leche smithemani*) which do not share grazing areas with cattle were sero-negative to *Brucella* ([Muma *et al.* 2011](#)).

The relatively high prevalence of brucellosis found at Clare B, Eglington and Share is quite worrisome since these areas are located more or less at the centre of the Mnisi area cushioned from the wildlife interface by other dip tank areas.

The absence of disease at Burlington, Islington, Ludlow A and Ludlow B can be attributed to the fact that these dip tanks previously formed part of the surveillance zone for FMD and were completely separated by a cordon fence from the rest of the dip tanks which formed part of the buffer zone for FMD. However, these dip tanks (Burlington, Islington, Ludlow A and Ludlow) became part of the buffer zone following a FMD outbreak in 2003.

The fence is still intact and movement of animals from or into these dip tank areas is still minimal and only occurs under a movement permit issued by the office of the local state veterinarian.

The outcome of the ELISA and CFT tests shows that both tests have almost the same ability to detect *B. abortus* antibody (12 of the 13 samples that tested positive for *B. abortus* on CFT also tested positive for *B. abortus* on ELISA). This demonstrates that the ELISA test is also a good confirmatory test for *B. abortus* antibody. A review of the sensitivity and specificity values of assays used for the detection of *B. abortus* antibody indicated that the ELISA test is more accurate than the conventional tests, RBT and CFT (Gall & Nielsen 2004). The ELISA test can therefore be recommended for use by Government Veterinary Services for routine brucellosis surveillance. The current national protocol uses RBT as a screening test followed by CFT as a confirmatory test.

The fact that *B. abortus* prevalence is relatively low means that brucellosis has a minimal influence on the health and fertility status of the Mnisi cattle population. This may also indirectly indicate that the Mnisi human population is at a relatively low risk of *B. abortus* infection from cattle. The possible occurrence of other production limiting diseases and conditions should be investigated.

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